

Micropropagation of three genotypes of Indian mustard [*Brassica juncea* (L.) Czern.] using seedling-derived transverse thin cell layer (tTCL) explants.

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Micropropagation of three genotypes of Indian mustard [*Brassica juncea* (L.) Czern.] using 7-days old seedling-derived transverse thin cell layer (tTCL) explants was accomplished.

The genotype, explant source and addition of silver nitrate to the medium significantly influenced shoot bud induction. MS medium with 26.6 μM of 6-Benzylaminopurin (BAP) and 3.22 μM of 1-naphtaleneacetic acid (NAA) was identical (in the case of cotyledon tTCLs whatever the organ) and superior for the induction of buds (in the cases of petiole tTCL explants of genotypes 1 and 2 and hypocotyl tTCL explants of genotypes 1 and 3) than 53.3 μM of BAP and 3.22 μM of NAA. However, 53.3 μM of BAP was superior for the induction of buds than 26.6 μM in the presence of the same concentration of NAA for petiole tTCL explants of genotype 3 and hypocotyl tTCL explants of genotype 2.

The addition of silver nitrate significantly enhanced the rate of shoot induction in all genotypes. Cotyledon-derived tTCL explants exhibited the highest shoot bud induction potential and was followed by petiole- and hypocotyl-derived ones. Addition of 10 μM of silver nitrate to BAP and NAA supplemented medium induced higher frequency shoot bud induction (up to 100 %) with the highest means of 4.45 shoots per cotyledon-derived tTCL explants obtained with the genotype 2. Shoot regenerated were rooted on MS basal medium without PGRs which induced 99 % of roots per shoot. The plantlets established in greenhouse conditions with 99 % survival, flowered normally and set seeds.

Keywords *Brassica juncea* - Genotype - Shoot regeneration – caulogenesis - Silver nitrate - transverse Thin Cell Layers (tTCLs).

Abbreviations BAP 6-Benzylaminopurin; NAA 1-naphtaleneacetic acid; AVG (aminoethoxyvinylglycin), tTCLs transverse Thin Cell Layers.

I. INTRODUCTION

Brassica oilseed crops including, *Brassica napus*, *B. rapa* and *B. juncea*, cover approximately 11 million hectares of the world's agricultural land and provide over 8% of the major oil grown under a variety of climatic conditions (Downey, 1990). The annual production of *Brassica juncea* in India alone reaches 5 million tons (FAO, 2003). *Brassica juncea* is widely grown as a vegetable but also for its uses in the food industry, in condiments, vegetable oils, hair industry, in lubricants and, in some countries, as a substitute for olive oil. Seed residues are used as cattle feed and in fertilizers (Reed, 1976).

For the sake of crop improvement, *Brassica* species have been subjected to many genetic manipulations with *Agrobacterium* species (Barfield and Pua, 1991; Jonoubi *et al.* 2005). However, it is better to obtain a high rate of shoot regeneration in these species for *in vitro* selection and routine genetic manipulation. Shoot regeneration in *Brassica juncea* has been reported from mesophyll protoplasts (Chatterjee *et al.* 1985), and traditional explants such as hypocotyls (Sethi *et al.* 1990), petioles (Pua and Chi, 1993), leaf discs and peduncle explants (Eapen and George, 1996, 1997), cotyledon or leaf segments (Guo *et al.* 2005) and microspores (Prem *et al.* 2005). However, to the best of our knowledge there is no report on shoot regeneration of *B. juncea* using explant-derived from young plants.

Shoot regeneration is affected by many factors like genotype, explant source, medium, growth hormones and physical conditions (Jain *et al.* 1988; Zhang *et al.* 1998; Tang *et al.* 2003; Guo *et al.* 2005).

In vitro culture of longitudinal thin cell layer explants, developed by Tran Thanh Van (1973), enabled regeneration

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in many species such as *Brassica napus* (Klimaszewska and Keller, 1985), *Beta vulgaris* (Detrez *et al.* 1988), *Lupinus mutabilis* and *L. albus* (Mullin and Bellio-Spataru, 2000).

The use of thin transverse sections has been successfully applied to *Panax ginseng* (Ahn *et al.* 1996), *Digitaria sanguinalis* (Bui *et al.* 1997), *Lilium longiflorum* (Nhut *et al.* 2002), *Spinacia oleracea* (Léguillon *et al.* 2003) or *Brassica napus* (Ben Ghnaya *et al.* 2008). Such technique with high reactivity and sensibility, seems to have a potential for intensive caulogenesis and regeneration of *Brassica juncea* plants.

In this study we investigate the effect of silver nitrate on the efficiency of shoot regeneration from cotyledon-, petiole- and hypocotyl-derived tTCL explants of three genotypes of *Brassica juncea* (L.) Czern.

II. MATERIALS AND METHODS

Plant material

Seeds of *Brassica juncea* I39/1 (genotype 1), AB79/1 (genotype 2) and J99 (genotype 3), provided by 'Ecole Nationale d'Enseignement Supérieur Agronomique (ENESA)' in Dijon (France) were used to evaluate shoot regeneration in presence or the absence of AgNO_3 . These genotypes are pure spring lines, genetically fixed and were obtained by autofertilization. The choice of concentrations of BAP and NAA is based on preliminary results in our laboratory that give relatively the best rate of shoot regeneration.

Culture conditions and plant regeneration

Seeds of *Brassica juncea* were decontaminated in 70% ethanol for 30 sec, followed by immersion in calcium hypochlorite (5%, w/v) added with two drops of Tween-20 for 10 min. The seeds were rinsed twice for 5 min with sterile water upon sterilization. For germination, one seed was placed per culture tube containing 0.6 % agar-solidified MS basal medium (Murashige and Skoog, 1962) and 2 % sucrose at 5.8 pH.

For the regeneration studies, tTCLs (400-500 μm thick) were excised from hypocotyls, petioles and cotyledons of 7 day-old plantlets. They were cultured in Petri dishes (15 tTCLs / dish) on MS medium supplemented with agar 0.6 % (w/v), sucrose 2% (w/v), NAA (3.22 μM), BAP (26.6-53.3 μM) and AgNO_3 (0-20 μM). All experiments were conducted in a culture chamber with a 12h photoperiod (60 $\mu\text{mol photon.m}^{-2}.\text{s}^{-1}$) provided by cool white fluorescent lamps with a 22/20°C thermoperiod (light/dark). The pH was adjusted to 5.8 with NaOH before autoclaving at 121°C for 20 min.

After three or four weeks, regenerated shoots were transferred to 0.7 % agar-solidified MS medium without any growth hormones. After two weeks, rooted plants were hardened under greenhouse conditions until flowering.

Data analysis

The number of explants that showed bud formation, as well as the number of buds per tTCL were counted and the frequency of bud formation was calculated.

There were 15 tTCLs per Petri dish and four plates per organ and treatment. Each experiment was repeated 3 times with 3 independent runs.

For data analysis, one way ANOVA and LSD (Least Significance difference) test were performed using the Statgraphics computer program at $P < 0.05$ or 0.01 (Statgraphics Plus version 5.1).

III. RESULTS

Results indicate that shoot regeneration ability is strongly influenced by genotype and tTCL explants between and within the *Brassica juncea* genotypes (Table 1). The first shoots were observed after ten days on tTCLs, whatever the genotype, and reached their largest number of occurrence at day-27. Callogenesis was the first observed

phenomenon, followed by rhizogenesis and caulogenesis respectively. Whatever the origin of tTCL explants, high rates of callogenesis and rhizogenesis were observed in calli culture (up to 99 %) (Figure 2 A, B and C). The caulogenesis frequency ranged from 0 to 1 % for genotype 3; 6.67 to 30.47 % for genotype 2 and 0 to 78.67 % for genotype 1. Furthermore, the origine of tTCL explants also affected shoot regeneration; cotyledon tTCLs best responded compared to petiole and hypocotyl tTCLs respectively and that whatever the genotype (Table 1). The best number of buds per tTCL explant was obtained with hypocotyl tTCLs of genotype 2 cultivated on MS medium added with 26.6 μ M BAP, 3.22 μ M NAA and sucrose 2% (w/v) (Table 2).

The presence of AgNO_3 in the culture medium showed a significantly beneficial effect on shoot regeneration. It influenced both the frequency of shoot regeneration (Table 1) and the number of buds per tTCL explant (Table 2). In MS medium added with 26.6 μ M BAP and 10 μ M AgNO_3 , *B. juncea* AB79/1 (genotype 2) does not show any significant difference between the frequencies of shoot formation between organs, which reach up to 100 % (Table 1). However, in the same conditions, the highest number of shoots per explant was obtained with hypocotyl tTCLs (Table 2).

In MS medium added with 26.6 μ M BAP and 10 μ M AgNO_3 , *B. juncea* I39/1 (genotype 1) and *B. juncea* J99 (genotype 3), cotyledon tTCLs were more sensitive for shoot regeneration than those of petiole and hypocotyl respectively (Table 1). Furthermore, their number of buds per tTCL explants rose from 1 to approximatively 2 when 10 μ M AgNO_3 were added to culture medium. The addition of 53.3 μ M BAP and 10 μ M AgNO_3 to culture medium seems to be more sensitive for given a highest frequency of shoot regeneration for petiole tTCL explants (19.07 %) than 26.6 μ M BAP (1.13 %) for genotype 3.

Moreover, 1 μ M AgNO_3 increases significantly caulogenesis for genotypes 1 and 2, but not for genotype 3. This more recalcitrant genotype required 10 μ M AgNO_3 to have a significant increase on bud formation (Table 1). Concentrations of AgNO_3 higher than 10 μ M (15 and 20 μ M) do not show any significant difference compared to 10 μ M whatever the genotype tested (data not shown).

In addition, in the presence of silver nitrate, calli were compact and had dark green color (Figure 1B). However, in its absence, calli were soft and had a pale green color whatever the concentration of growth regulators and the origin of tTCL explants (Figure 1A).

Finally, after 27 days of tTCL culture, shoots excised and transferred in the test tubes on 0.7 % agar-solidified MS medium without PGRs exhibited rooting and rapid development (Figure 2D). After 2 weeks, plantlets were transferred to grow in pots under greenhouse conditions. The phenotype of regenerant plants were similar to plant control grown from seeds. They flowered normally 6 weeks after transfer in pots and set seeds (Figure 2E).

IV. DISCUSSION AND CONCLUSION

Thin cell layer technology was known efficient for the propagation of various plant species. This study was conducted to achieve a high rate of regeneration in *B. juncea* L. Czern. From tTCL explants in presence or in the absence of silver nitrate. This technique combined to AgNO_3 promoted rapid and high frequency of shoot regeneration with shoot buds developing within 10 days for all *B. juncea* genotypes.

These results compare favourably with recent studies of shoot regeneration of *B. napus* L. from traditional explants (Tang *et al.* 2003; Akasaka-Kennedy *et al.* 2005), longitudinal thin cell layers (Klimaszewska and Keller, 1985) and transverse thin cell layers (Ben Ghnaya *et al.* 2008). In our study, tTCL explants were excised transversally from 7-day old axenic plants.

In our experiments, all factors evaluated (genotype, explant, BAP and AgNO_3) influenced shoot regeneration. Shoot regeneration ability is strongly influenced by genotype as proved earlier in *B. napus* L. (Ono *et al.* 1994; Akasaka-Kennedy *et al.* 2005; Ben Ghnaya *et al.* 2008) and *B. campestris* L. ssp. *Pekinensis* (Zhang *et al.* 1998). *B. juncea* AB79/1 (genotype 2) and *B. juncea* I39/1 (genotype 1) showed a greater capacity to produce shoots on the MS medium containing 3.22 μ M NAA, 26.6 or 53.3 μ M BAP and sucrose 2 % (w/v) than *B. juncea* J99 (genotype 3). Furthermore, we showed an explant effect on shoot regeneration process. For all genotypes, cotyledon tTCLs best responded than petiole and hypocotyl tTCLs and exhibited the highest shoot regeneration rate (Table 1). Tang *et al.* (2003) and more recently Ben Ghnaya *et al.* (2008) showed that PGR content affected significantly the regeneration

process as observed from tTCL explants in our study. Whatever the genotype and organ, 26.6 μM BAP seems to be more sensitive on shoot regeneration frequency than 53.3 μM .

Moreover, silver nitrate showed to be significantly beneficial to the shoot regeneration process for all genotypes of *Brassica juncea*, even for the more recalcitrant one (genotype 3). Indeed, 10 μM AgNO_3 was able to induce the response of genotype 3 but concentrations up to more than 10 μM (15 and 20 μM , data not shown) didn't increase the frequencies of caulogenesis (Table 1). The positive effect of silver nitrate on shoot regeneration process, was shown in previous studies with traditional explants such as cotyledons of *Brassica rapa* ssp. *Oleifera* (Burnett *et al.* 1998), *Brassica campestris* ssp. *Pekinensis* (Chi *et al.* 1991; Zhang *et al.* 1998), hypocotyls of *Brassica juncea* (Pua and Chi, 1993), peduncle and leaf segments of *Brassica napus* (Eapen and George, 1997; Akasaka-Kennedy *et al.* 2005). Furthermore, the presence of silver nitrate, especially 10 μM seems to be sensitive significantly in the enhancement of the number of buds per tTCL explants, whatever the genotype and the organ (Table 2).

In the present study, an original and efficient regeneration system from cotyledon, petiole and hypocotyl tTCLs has been developed in *Brassica juncea*. It produces good results with tTCL explants of all genotypes. Despite a smaller surface and a larger number of wounded cell, shoot regeneration is obtained typically ten days after the tTCL explant initiation culture. This swift response, in agreement with the observation described previously by Tran Thanh Van (1973), due to the combined process of cell dedifferentiation and reprogramming. Typically, two months later, we observed the normal flowering of regenerant plantlets. Nonetheless, in this system, a single subculture step preceding the regenerated plant transfer into culture tube is required and no subsequent phenotypic alterations were observed in these plants at all.

For further improvements, other factors could be taken into account, such as the concentration of sugar used (Ben Ghnaya *et al.* 2008), the use of other inhibitors that block ethylene synthesis (e.g. AVG, nickel and cobalt) or action (silver thiosulfate) (Chraïbi *et al.* 1991; Burnett *et al.* 1994), but also tTCL explant thickness or position along the organ (Nhut *et al.* 2001).

Our tTCL model could be used as a tool for fundamental regeneration studies and for crop improvement using *Agrobacterium* transformation of *Brassica juncea* cultivars as well as other *Brassica* species. Furthermore this system may will be used for *in vitro* selection, in presence of many metals, of plants which may will be used in different phytoremediation processes.

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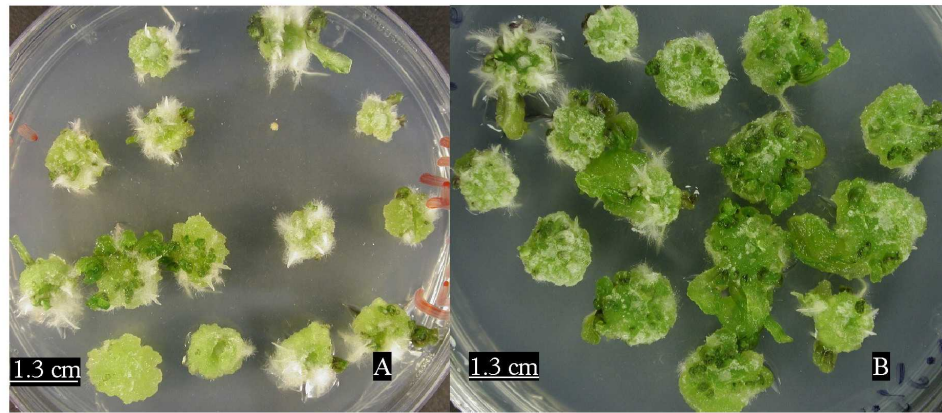


FIG. 1: Morphology of calli obtained from hypocotyl tTCL explants of *Brassica juncea* AB79/1 in absence (A) or in presence (B) of AgNO_3 .



FIG. 2: Shoot regeneration from tTCL explants of *Brassica juncea* AB79/1. (A): cotyledon tTCLs; (B): petiole tTCLs; (C): hypocotyl tTCLs; (D): plantlet obtained from excised tTCL grown 2 weeks in a test tube and (E): regenerated plants grown and flowered in the greenhouse.

The frequency of shoot regeneration were recorded 27 days after the tTCL initiation culture on MS basal medium supplemented with NAA ($3.22 \mu\text{M}$), BAP (26.6 or $53.3 \mu\text{M}$), sucrose 2 % (w/v) and AgNO_3 ($0 - 10 \mu\text{M}$). The results were calculated from three independent experiments, each with, at least, five Petri dishes with 15 tTCLs per dish. The mean values with different letters are significantly different at $p = 0.05$ (LSD test).

TABLE I: Effect of genotype, explant, BAP and AgNO₃ on *in vitro* organogenesis from tTCLs of cotyledons, petioles and hypocotyls. The percentage of buds were recorded 27 days after the tTCL initiation culture.

Genotypes	AgNO ₃	BAP	Frequency of of regenerating tTCL (%)		
			Cotyledons	Petioles	Hypocotyls
1	0	26.6		78.67 ^c	23.34 ^{fg}
				93.34 ^a	68.93 ^d
				96.67 ^a	75.60 ^c
				97.34 ^a	77.80 ^c
	5	26.6			13.34 ^{gh}
	10	26.6			18.67 ^g
	0	53.3		72.20 ^c	8.00 ^{gh}
				86.67 ^b	32.27 ^f
				93.34 ^a	44.00 ^e
				94.47 ^a	46.67 ^e
2	0	26.6		30.47 ^f	22.27 ^{fg}
				93.34 ^a	87.88 ^b
				96.67 ^a	92.27 ^a
				100.00^a	98.87 ^a
	5	26.6			92.23 ^a
	10	26.6			
	0	53.3		26.67 ^f	10.00 ^{gh}
				95.34 ^a	67.80 ^d
				95.60 ^a	75.60 ^c
				96.67 ^a	84.47 ^b
3	0	26.6		0.93 ^c	0.00 ^k
				0.93 ^c	0.00 ^k
				6.67 ^h	9.53 ^{gh}
				15.20 ^g	9.53 ^{gh}
	5	26.6			
	10	26.6			
	0	53.3		0.93 ^j	0.93 ^j
				0.93 ^j	0.00 ^k
				12.40 ^{gh}	10.47 ^{gh}
				13.34 ^{gh}	19.07 ^g

Data were recorded 4 weeks after the tTCL initiation culture on MS basal medium supplemented with NAA (3.22 μ M), BAP (26.6 or 53.3 μ M), sucrose 2 % (w/v) and AgNO₃ (0 - 10 μ M).

The results are obtained from three independent experiments, each with, at least, five Petri dishes and 15 tTCLs per dish. Letters indicate significant statistical differences at $p = 0.01$ (One way ANOVA and LSD test).

TABLE II: Effect of genotype, explant, BAP and AgNO₃ on the number of adventitious buds per tTCL excised from different organs (cotyledons, petioles and hypocotyls).

Genotypes	AgNO ₃	BAP	Number of buds per tTCLs		
			Cotyledons	Petioles	Hypocotyls
1	0	26.6		1.34 ^{ef}	1.16 ^{ef} 0 ^g
	1			1.69 ^e	1.75 ^e 1.00 ^f
	5			1.81 ^e	1.86 ^e 1.07 ^f
	10			1.86 ^e	1.90 ^e 1.12 ^f
	0	53.3		1.26 ^{ef}	1.00 ^f 0 ^g
	1			1.51 ^e	1.02 ^f 1.00 ^f
	5			1.82 ^e	1.23 ^{cd} 1.00 ^f
	10			1.89 ^e	1.30 ^{cd} 1.14 ^f
	0	26.6		1.00 ^f	1.23 ^{ef} 2.09 ^{de}
	1			1.64 ^e	2.60 ^d 2.88 ^d
	5			2.27 ^{de}	3.16 ^c 3.87 ^b
	10			2.40 ^{de}	3.30 ^c 4.45^a
2	0	53.3		1.00 ^f	1.12 ^f 1.42 ^{ef}
	1			1.14 ^f	2.75 ^d 1.68 ^e
	5			2.15 ^{de}	3.26 ^c 2.67 ^d
	10			2.22 ^{de}	3.43 ^c 2.78 ^d
	0	26.6		1.00 ^f	0 ^g 0 ^g
	1			1.00 ^f	0 ^g 1.00 ^f
	5			1.23 ^{ef}	0 ^g 1.12 ^f
	10			1.34 ^{ef}	1.00 ^f 1.23 ^{ef}
	0	53.3		1.00 ^f	1.00 ^f 0 ^g
	1			1.00 ^f	1.11 ^f 0 ^g
	5			1.27 ^{ef}	1.31 ^{ef} 0 ^g
	10			1.41 ^{ef}	1.37 ^{ef} 0 ^g
3	0	26.6		1.00 ^f	0 ^g 0 ^g
	1			1.00 ^f	0 ^g 1.00 ^f
	5			1.23 ^{ef}	0 ^g 1.12 ^f
	10			1.34 ^{ef}	1.00 ^f 1.23 ^{ef}
	0	53.3		1.00 ^f	1.00 ^f 0 ^g
	1			1.00 ^f	1.11 ^f 0 ^g
	5			1.27 ^{ef}	1.31 ^{ef} 0 ^g
	10			1.41 ^{ef}	1.37 ^{ef} 0 ^g